EXHIBIT 32

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5′-Terminal Caps, Cap-Binding Proteins and Eukaryotic mRNA Function

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Introductory Overview of mRNA Modification

Eukaryotic mRNAs are easily distinguished from other RNAs, including prokaryotic messengers, by the presence of one or more unique structural features. They include methylated adenosine in internal positions, 3′-terminal polyadenylic acid and 5′ ends that are 'capped' by 7-methylguanosine. All three kinds of modifications occur in the nucleus on RNA polymerase II pre-mRNA transcripts of both cellular and viral origin (for reviews, see Revel & Groner, 1978; Darnell, 1979; Shenk, 1981). By contrast, animal viruses that replicate in the cytoplasm of infected cells produce mRNAs that usually are not methylated at internal sites (reviewed by Banerjee, 1980). Furthermore, some functional viral mRNAs (reovirus, frog virus 3) are not 3′-polyadenylated (Stoltzfus *et al.*, 1973; Willis & Granoff, 1976), and others (picornaviruses) are devoid of a 5′-terminal cap (Flanegan *et al.*, 1977; Lee *et al.*, 1977). From these diverse observations, the biological significance of mRNA methylation and terminal modification for eukaryotic genetic expression is not readily apparent.

Some eukaryotic genes, e.g. those coding for cellular histones (Kedes, 1979) and vaccinia virus proteins (Wittek *et al.*, 1980), are notable for the absence of intron sequences. The corresponding transcripts also are not methylated internally (Kedes, 1979, Moss *et al.*, 1977), and it therefore seemed reasonable to suggest that methylated adenosine acts as a recognition signal during mRNA splicing. However, studies with methylation inhibitors have indicated that processing and intracellular transport of mRNA, like translation, are probably not strictly dependent on internal methylation (Kaehler *et al.*, 1977; Dimock & Stoltzfus, 1978, 1979; Bachellerie *et al.*, 1978). Thus a biochemical role for methyladenosine in mRNA remains to be demonstrated.

The importance of 3′-polyadenylation for eukaryotic mRNA formation and function is somewhat clearer. Addition of poly(A) to cleaved nascent transcripts is a primary maturation event that defines mRNA 3′ ends (Hofer & Darnell, 1981; Fitzgerald & Shenk, 1981). A stabilizing effect of 3′-poly(A) on cytoplasmic mRNA has been described recently (Zeevi *et al.*, 1981). In addition, the presence

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of 3′-poly(A) is apparently important for the long-term translation of globin mRNA in microinjected *Xenopus* oocytes (Huez *et al.*, 1975) and for reinitiation of ovalbumin synthesis in reticulocyte lysate (Doel & Carey, 1976).

One of the recently discovered and intensely studied eukaryotic mRNA modification events is 5′-terminal capping (Shatkin, 1976). Cap structures of the type $m^7G(5')ppp(5')N^m pNp\ldots$ (Fig. 1) have been detected on short pre-mRNA transcripts. This strongly suggests that capping is an early event in cellular mRNA synthesis (Salditt-Georgieff *et al.*, 1980). Studies of viral systems also indicate that capping probably occurs as part of the initiation of transcription. Caps are retained during transcript processing, and in various systems their presence has been found to influence gene expression at the levels of transcription, translation and mRNA stability.

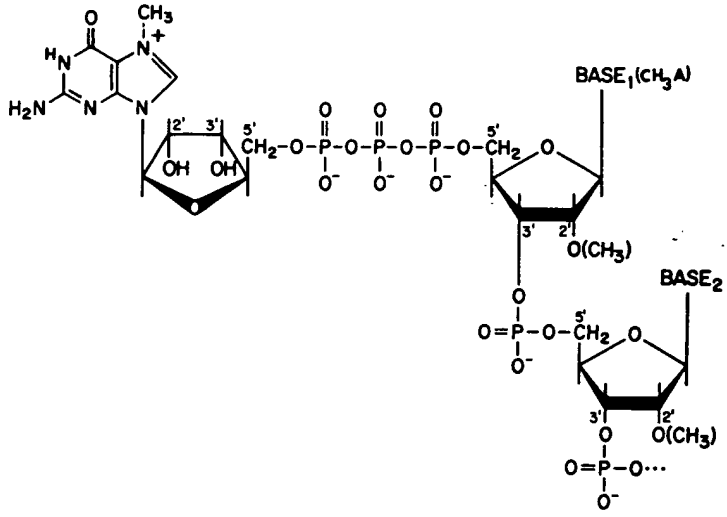

Fig. 1. *General structure of 5′-terminal cap of eukaryotic mRNAs*

The terminus invariably is m^7G. Caps of some mRNAs (e.g. from plants, plant viruses, lower eukaryotes including yeast) have no additional methyl groups and are referred to as cap zero. Most others contain a 2′-*O*-methylated penultimate residue, i.e. a cap one structure. If the base 1 is adenine, it may be ring-methylated in the *N*-6 position. A cytoplasmic 2′-*O*-methylating activity can also modify base 2 to form a cap two structure.

Cap Synthesis and Transcription Initiation

The biochemical mechanism of cap formation has been elucidated by studying the complex of enzymes associated with purified reovirus (Furuichi *et al.*, 1976) and vaccinia virus (Moss *et al.*, 1976). Capping enzymes isolated from HeLa cell nuclei (Venkatesan *et al.*, 1980*a*) and from purified vaccinia virus (Venkatesan *et al.*, 1980*b*; Shuman *et al.*, 1980) catalyse the same series of reactions. They include:

(1) RNA polymerase — $pppN + pppN \rightarrow pppNpN\ldots + PP_i$

(2) Nucleotide phosphohydrolase — $pppNpN\ldots \rightarrow ppNpN\ldots + P_i$

(3) Guanylyltransferase $\quad ppNpN... + ppp\overset{*}{G} \rightleftarrows G\overset{*}{p}pppNpN... + PP_i$

(4) Methyltransferase 1

$G\overset{*}{p}pppNpN... +$ *S*-adenosylmethionine $\rightarrow m^7G\overset{*}{p}pppNpN... +$ *S*-adenosylhomocysteine

(5) Methyltransferase 2

$m^7G\overset{*}{p}pppNpN... +$ *S*-adenosylmethionine $\rightarrow m^7G\overset{*}{p}pppN^mpN... +$ *S*-adenosylhomocysteine

The reovirus-associated guanylyltransferase and the corresponding activities purified from HeLa cells and vaccinia virus are capable of capping dinucleotides (reaction 3 above). The enzymes can also use polynucleotides containing 5'-diphosphate ends as guanylate acceptors. Consequently the exact time of cap addition to pre-mRNA transcripts *in vivo* is difficult to establish. What seems clear from recent analyses of the reovirion capping enzymes is that all five of the above reactions can be carried out in the absence of chain elongation. This is illustrated by the results shown in Fig. 2. Reovirus cores derived from virions by chymotrypsin digestion

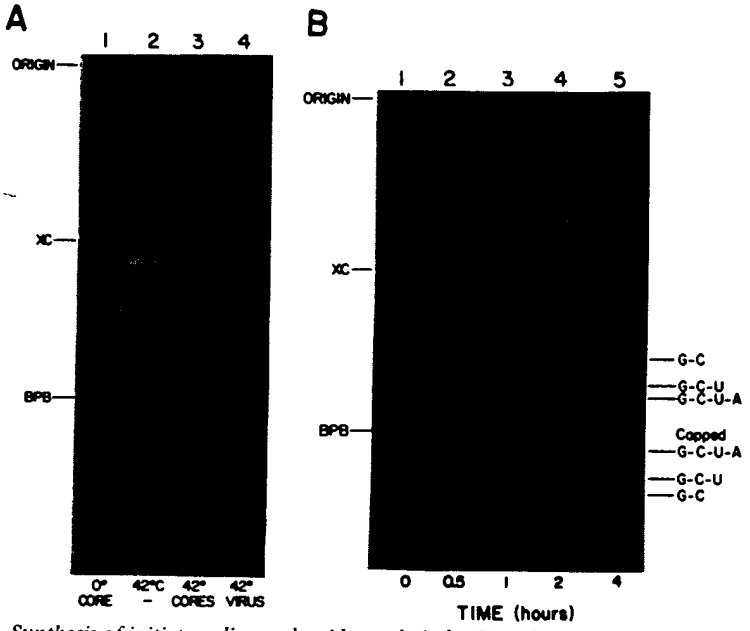

Fig. 2. *Synthesis of initiator oligonucleotides and viral mRNA by reovirus-associated enzymes*

A. Transcription reaction mixtures (25 μl) containing [α-^{32}P]CTP as the radioactive precursor and reovirions (25 μg) or viral cores derived from them by chymotrypsin digestion were incubated for 30 min, and the resulting products were analysed by electrophoresis in a 20% polyacrylamide gel as described previously (Yamakawa *et al.*, 1981). mRNA synthesized by cores remained at the origin. Initiator oligonucleotides that migrated faster than the marker dyes xylene cyanol (XC) and bromphenol blue (BPB) were eluted and analysed further by chromatography and electrophoresis after enzymic digestions. Capped and uncapped structures corresponding to the 5'-terminal sequence of viral mRNA were identified, e.g. m^7GpppG^mpC, $m^7GpppGpC$, GpppGpC and ppGpC. B. Virion transcriptase products labelled with [α-^{32}P]CTP and *S*-adenosyl[*methyl*-^{3}H]methionine were collected after 0, 0.5, 1, 2, and 4 h of incubation (lanes 1–5, respectively). Samples were digested with alkaline phosphatase before gel analysis. Material migrating in the positions of the capped oligonucleotides was eluted and analysed further as described in the legend to Table 1.

synthesize capped viral mRNAs *in vitro*. They also produce a high molar excess of short 'initiator' oligonucleotides that correspond in sequence to mRNA 5′-termini (panel A, lane 3). Intact reovirions produce the same capped and uncapped oligonucleotides, indicating that they contain the capping enzymes in an active form (lane 4). Although initiation and capping of nascent oligonucleotides as short as dinucleotides are effectively carried out by whole virions, no mRNA is produced even during an incubation period of several hours (panel B). Apparently elongation by the virion RNA polymerase is prevented by structural constraints on the intact particles. Analyses of the relative proportions of GpppG, m^7GpppG and m^7GpppG^m at the 5′-ends of the capped oligonucleotides synthesized by the reovirion enzymes is consistent with the order of reactions shown above (Table 1). These results indicate that capping accompanies initiation of transcription *in vitro*. Studies on adenovirus mRNA formation in infected cells have also demonstrated that caps are added early to initiated 5′-ends of incomplete transcripts (Babich *et al.*, 1980). Thus capping occurs before extensive chain growth and presumably precedes other modifications including internal methylation and polyadenylation as well as splicing. The early addition of caps to nuclear transcripts and their retention during mRNA maturation and passage to the cytoplasm is consistent with a protective effect of blocked 5′-termini on eukayotic mRNAs (Furuichi *et al.*, 1977).

A functional relationship between initiation of transcription and capping has been described for the virion-associated RNA polymerases in purified insect cytoplasmic polyhedrosis virus (Furuichi, 1978) and influenza virus (Krug, 1981). The number of starts made by the polyhedrosis virus transcriptase is markedly increased by the presence of *S*-adenosylmethionine. The methyl donor as well as some of its structural analogues are positive allosteric effectors of transcription initiation. They act by lowering the K_m for ATP, the initiating nucleotide for the RNA polymerase. Apparently the methyltransferase and RNA polymerase are structurally coupled in the virion nucleoprotein complex, and binding of methyl donor by the transferase results in activation of the polymerase.

The mechanism of influenza virus mRNA synthesis is unusual. The virion polymerase uses capped heterologous polynucleotides, i.e. nascent cellular pre-mRNAs in infected cells, to prime viral mRNA formation. An endonuclease that is present

Table 1. *Composition of capped virion oligonucleotide products*

Capped oligonucleotides eluted in water from the gel shown in Fig. 2B were counted, desalted and digested with P_1 nuclease (Furuichi *et al.*, 1976). Cap structures were separated by paper chromatography in isobutyric acid/0.5 M-NH_4OH (10 : 6, v/v) and localized by counting the paper cut into 1 cm strips. The caps were extracted and digested with venom phosphodiesterase followed by alkaline phosphatase; the resulting 7-methylguanosine and 2′-*O*-methylguanosine were separated by high voltage paper electrophoresis. Total and relative amounts of each of the capped oligonucleotides were calculated on the basis of the specific activities of the [α-^{32}P]CTP and *S*-adenosyl[*methyl*-^{3}H]methionine used in the reaction mixture.

		Composition (%)		
Oligonucleotide	Total (pmol)	GpppG	m^7GpppG	m^7GpppG^m
Capped G-C	0.093	78.5	17.6	3.9
Capped G-C-U	0.061	63.9	31.7	4.4
Capped G-C-U-A	0.053	92.4	6.1	1.5

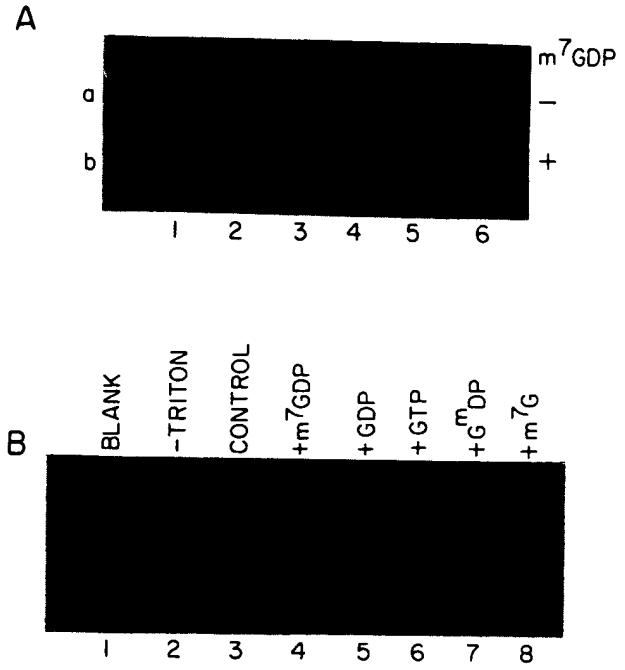

Fig. 3. *Cap binding activity of cellular (A) and influenza virus (B) proteins detected by filter blotting*

m^7GpppG^mpC was obtained from capped reovirus mRNA by RNAase T2 and alkaline phosphatase digestion followed by high voltage paper electrophoresis. The purified caps were radiolabelled to a specific activity of 1–2 μCi/pmol by incubation with [^{32}P]pCp and T4 RNA ligase. After phosphatase treatment and repurification, the $m^7GpppG^mpC^*pC$ was used as an affinity probe for cap binding activity. Protein samples in 2 μl of binding buffer [20 mM-Hepes (pH 7.4)/70 mM-KCl/3 mM-magnesium acetate/1 mM-dithiothreitol) were spotted on strips of cellulose nitrate paper (BA85, S & S) which had been soaked for 10 min in the buffer. To each protein spot was applied 2 μl of $m^7GpppG^mpC^*pC$ in water with or without 0.5 mM-m^7GDP. The strip was soaked for 5 min at room temperature, washed three times in 100 ml each of the buffer, dried and the spots were located by autoradiography (Kroath & Shatkin, 1982). A. Samples of rabbit erythrocyte cap binding protein eluted from m^7GDP–Sepharose affinity resin (Sonenberg *et al.*, 1979) with m^7GDP (spots 1–3) and subsequently with 1 M-KCl (spots 4–6) were tested with 0.04 pmol (27000 c.p.m.) of $m^7GpppG^mpC^*pC$. Protein applied (in ng): spot 1, 360; 2, 140; 3, 340; 4, 560; 5, 590; 6, 580. Exposure 20 h; radioactivity bound in spot 3a = 6300 c.p.m. and in 3b = 140 c.p.m. B. Purified influenza A virus strain WSN (kindly provided by Dr. Robert Krug) was incubated in a transcription reaction mixture (11 μg in 25 μl) containing 50 mM-Tris/HCl (pH 7.8)/1 mM-dithiothreitol/120 mM-potassium acetate/5 mM-magnesium acetate/1 mM of each rNTP and 0.4% Triton N101. After 10 min at 31°C, 2 μl aliquots were assayed as in A but with 0.11 pmol (46000 c.p.m.) of $m^7GpppG^mpC^*pC$ and 0.1 mM of the indicated compounds. Spot 1 is + Triton, – virus; 2 is – Triton, + virus; 3–8 are + Triton, + virus. Exposure was 24 h.

in virions cleaves the foreign RNAs at a measured distance (approx. 10–15 nucleotides) from the cap. The polymerase utilizes the capped fragments as primers to transcribe the influenza RNA templates. Thus the resulting mRNAs are chimaeric and consist of a newly formed influenza sequence joined to a derived, heterologous 5'-capped leader. The dependence of this series of unique events on a preformed cap suggests that influenza virus contains cap recognizing protein(s). This is further

indicated by the ability of influenza virus, like cellular cap binding protein, to bind ^{32}P-labelled cap structures as detected by a filter blotting assay (Fig. 3). Binding depends on detergent activation of the virus and is inhibited by cap analogues. It is not a general property of viruses that contain transcriptases since vaccinia and reoviruses synthesize capped viral mRNAs entirely *de novo* but do not have detectable cap binding activity (Table 2). Association of cap binding activity with an influenza virion P protein has recently been demonstrated by using radiolabelled cap analogues (Blaas *et al.*, 1981) and capped polynucleotides as photoaffinity probes (Ulmanen *et al.*, 1981). It remains to be determined if this influenza protein is related to cellular cap binding proteins, but influenza transcription primed by capped mRNA *in vitro* is inhibited by cap binding protein purified from rabbit erythrocytes (Kroath & Shatkin, 1982).

Table 2. *Binding of $m^7GpppG^mpC\overset{*}{p}C$ to purified viruses*

Purified influenza virus, vaccinia virus, and reovirus (11 μg, 18 μg and 25 μg of protein, respectively), were incubated for 10 min at 31°C in transcription mixtures as in Fig. 3B. Aliquots of 2 μl were removed and tested with 0.06 pmol (23000 c.p.m.) of ^{32}P-labelled caps in the presence and absence of 0.4 mM-m^7GDP. Spots located by autoradiography were cut out and counted in toluene-based scintillant.

Additions		Binding (c.p.m.)		
		Influenza	Vaccinia	Reo
Triton	m^7GDP			
−	−	46	33	47
−	+	24	23	11
+	−	333	17	28
+	+	13	10	6

Fascinating problems remain to be solved concerning the enzymic mechanisms of cap formation, the spatial configurations assumed by caps, and the functional interactions of caps during mRNA maturation and function. One important functional effect of caps is its facilitating effect on protein synthesis. The enhancement appears to occur at the level of initiation. Recent findings suggest that cap binding protein(s) are involved in promoting stable interactions between 40 S ribosomal subunits and 5′-cap-vicinal sequences in eukaryotic mRNAs.

Role of 5′-Cap in the Initiation of Translation: Reversible Inhibition by Cap Analogues

Many different kinds of experiments have provided evidence that the presence of a 5′-terminal cap enhances eukaryotic mRNA translation (Shatkin, 1976; Filipowicz, 1978; Banerjee, 1980). For example, removal of caps by chemical or enzymic treatment of viral and cellular mRNAs diminished their capacity to bind to ribosomes in cell-free systems and to direct protein synthesis in microinjected *Xenopus* oocytes. Furthermore, capped reovirus mRNAs effectively formed stable initiation complexes *in vitro* while the corresponding uncapped RNAs did not. Ribosomal subunits initially attached to reovirus mRNAs at or near the cap, as shown by 40 S

subunit protection of mRNA 5′-sequences, including the 5′-proximal AUG, against digestion by RNAase. In most other viral and cellular mRNAs the 5′-proximal AUG also serves as the initiator codon for polypeptide synthesis (Kozak, 1981).

Involvement of the cap in the initiation of translation is probably mediated or at least influenced by cap binding protein(s). A protein of M_r approx. 24000 that binds to caps (24K-CBP) has been purified from rabbit reticulocyte ribosomal salt wash by affinity column chromatography on m^{7}GDP-Sepharose (Sonenberg *et al.*, 1979). It stimulated the translation *in vitro* of capped but not of uncapped mRNAs (Sonenberg *et al.*, 1980). Binding of the 24K-CBP to mRNA 5′-ends was competed out by the presence of an excess of cap analogues such as m^{7}GMP and m^{7}GDP. The same kinds of compounds added to cell-free translating systems prevented ribosome binding to capped mRNAs. This is illustrated in Fig. 4. Reovirus mRNA formed 80 S initiation complexes in wheat germ extract (panel A), and the presence of 1 mM-m^{7}GMP inhibited binding by 83% (panel B). One interpretation of these results is that cap binding protein(s) interact with mRNA 5′-termini and promote conformation(s) favourable for attachment of ribosomal subunits.

It was suggested earlier from a comparison of three-dimensional structure and translation inhibitory activity that active cap analogues (and by inference capped mRNA 5′ ends) assume a 'rigid' conformation that is preferred for initiation of

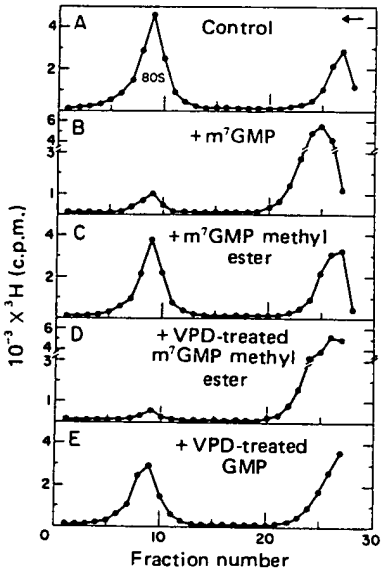

Fig. 4. *Cap analogue effects on ribosome binding*

Capped, 5′-[^{3}H]methyl-labelled reovirus mRNA was synthesized *in vitro* by incubating viral cores in transcription reaction mixtures containing *S*-adenosyl[*methyl*-^{3}H]methionine (Muthukrishnan *et al.*, 1976). The purified mRNA was incubated in wheat germ 'S23' extract in the presence of 0.2 mM-sparsomycin under conditions of 80S initiation complex formation but not polypeptide chain elongation. After 10 min at 25°C complexes were analysed by glycerol gradient centrifugation. Incubation mixtures contained 1 mM-m^{7}GMP (B) or the m^{7}GMP methyl ester without treatment (C) or after digestion as in Fig. 6 with venom phosphodiesterase (D) or venom plus calf intestine alkaline phosphatase (E).

protein synthesis (Hickey *et al.*, 1977; Adams *et al.*, 1978). The rigidity of $m^7Gp(p)\ldots$ apparently results from an electrostatic interaction between the positively charged imidazole portion of the N-7 alkylated guanosine and the negative phosphate group(s) (see Fig. 1). To test the proposed correlation between preferred conformation and cap function, the methyl ester of m^7GMP was chemically synthesized. This cap analogue was predicted to have a decreased negativity due to the availability of only one, rather than two, hydroxyls on the phosphate group (Fig. 5). N.m.r. spectroscopy studies indicated that the conformations of m^7GMP and m^7GMP methyl ester were very similar (Darzynkiewicz *et al.*, 1981). However, other properties of the two compounds were strikingly different. For example, the methyl ester was resistant to attack by alkaline phosphatase while m^7GMP was converted to the positively charged nucleoside by phosphatase digestion (Fig. 6, lanes 5 and 3). Treatment with venom phosphodiesterase hydrolysed the phosphate-

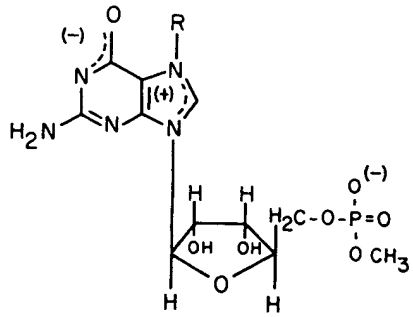

Fig. 5. *Structure of 7-methylguanosine-5'-phosphate methyl ester*

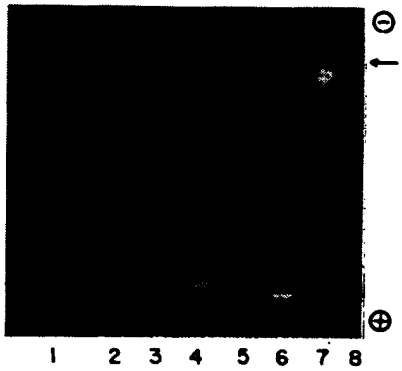

Fig. 6. *Susceptibility of m^7GMP methyl ester to enzyme digestion*

After paper electrophoresis in pyridine/acetate buffer (pH 3.5) the fluorescent samples were photographed under u.v. light. Lanes 1 and 8 are 10 nmol of m^7G. Lanes 2, 3 are 50 nmol of m^7GMP before and after digestion with calf intestine alkaline phosphatase [20 units/ml in 0.02 M-Tris/HCl (pH 8), 37°C, 30 min]. Other lanes are 50 nmol of m^7GMP methyl ester analysed directly (4) or (5) treated with phosphatase as in lane 3 or (6) digested with venom phosphodiesterase [2 units/ml in 0.02 M-Tris/HCl (pH 8), 10 mM-magnesium acetate, 0.1 M-NaCl, 37°C, 30 min] and repeated, followed by 2 min at 50°C to inactivate the enzyme and (7) treated sequentially with phosphodiesterase and phosphatase but at 30 units of calf phosphatase/ml.

methyl ester, and the resulting m^7GMP could then be converted to m^7G by incubation with phosphatase (lanes 6 and 7).

These enzyme susceptibility results were reflected in functional tests of cap analogue activity as shown in Fig. 4. In contrast to the strong inhibitory activity of 1 mM-m^7GMP on ribosome binding (panel B), the same concentration of methyl ester had little effect on translation initiation as measured by 80 S initiation complex formation (panel C). Conversion of the m^7GMP methyl ester to m^7GMP by venom phosphodiesterase digestion was accompanied by acquisition of inhibitory activity (panel D). Restoration of cap analogue activity was not due to a non-specific effect (e.g. the presence of an inhibitory contaminant) because sequential digestion of m^7GMP methyl ester with phosphodiesterase and phosphatase yielded the non-inhibitory nucleoside product, m^7G (panel E). These findings indicate that although the rigid conformation of m^7GMP (as determined from n.m.r. measurements) was retained following methyl esterification, the cap analogue activity of the esterified m^7GMP was lost. Thus a rigid 5'-terminal conformation may be necessary, but clearly it is not sufficient for cap recognition during initiation of translation.

The inhibitory activity of m^7GMP on ribosome binding may be due to competition with mRNA for protein(s) that recognize and bind to the 5'-cap during initiation. To test this possiblity a chemical cross-linking procedure was used as described in detail previously (Sonenberg *et al.*, 1978). In this method affinity purified 24K-CBP was radiolabelled by reductive cross-linking to periodate-oxidized, 5'-^{3}H-labelled mRNA. Cap-specific interaction of the 24K-CBP with reovirus mRNA was progressively inhibited by increasing concentrations of m^7GMP. At 0.25 mM the extent of inhibition was 85% (Fig. 7, lane 3). As also observed in the ribosome binding assay (Fig. 4C), the methyl ester was far less inhibitory, and a level of 0.25 mM decreased 24K-CBP radiolabelling by only 11% (Fig. 7, lane 7). The results provide evidence for a direct effect of cap analogues on the interaction of 24K-CBP with mRNA 5' ends. They suggest further that the presence of an extra methyl group on the phosphate moiety of m^7GMP perturbs the 'fit' between cap and the binding protein(s) involved in initiation complex formation.

Capped mRNA translation *in vitro* is increased by addition of purified 24K-CBP, and it was of interest to determine if this enhancement was affected by the presence of cap analogues. HeLa cell-free translating extracts synthesized mainly viral capsid protein in response to Sindbis virus mRNA. The yield of this M_r 33000 polypeptide was increased several-fold by addition of affinity purified 24K-CBP (Fig. 8, lanes 1–3). The increase was partially prevented by 0.1 mM m^7GMP (Fig. 8, lane 5), and a higher concentration was almost completely inhibitory (lane 4). Consistent with results obtained in the other assays for cap analogue activity, 0.25 mM-m^7GMP methyl ester had little effect on translation of Sindbis mRNA in HeLa cell extracts (Fig. 8, lanes 7–9). These and other data taken together provide strong evidence for a functional interaction between mRNA 5'-caps and 24K-CBP during initiation of translation.

Translation Inhibition by Antibody to 24K-CBP

A functional role of 24K-CBP in protein synthesis was also suggested by the results of studies with a monoclonal antibody to this polypeptide. Antibody was

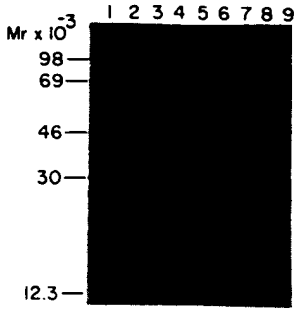

Fig. 7. *Cap analogue inhibition of cap binding protein interaction with mRNA as measured by chemical cross-linking*

Each sample contained 2.3 μg of [^{3}H]methyl-labelled, periodate-oxidized reovirus mRNA (specific radioactivity 10^5 c.p.m./μg) and 1.8 μg of 24K-CBP purified from rabbit erythrocytes by affinity chromatography. Binding was done (Sonenberg *et al.*, 1979) in the absence (lane 1) or presence of 0.5, 0.25, 0.1, or 0.05 mM-m^{7}GMP (lanes 2–5, respectively) or the same concentration of m^{7}GMP methyl ester (lanes 6–9, respectively). After $NaBH_3CN$ reduction and RNAase digestion, proteins radiolabelled by covalently bound [^{3}H]caps transferred from oxidized mRNA were detected by polyacrylamide gel electrophoresis and fluorography. Films were traced, and the normalized levels of crosslinking based on relative densities of the approx. 24 kDa band in lanes 1–9 were: 1.0, 0, 0.15, 0.31, 0.15, 0.38, 0.89, 0.78, and 1.12, respectively. Values obtained with control samples that contained GMP and GMP methyl ester (0.5 mM) were 1.19 and 1.25, respectively.

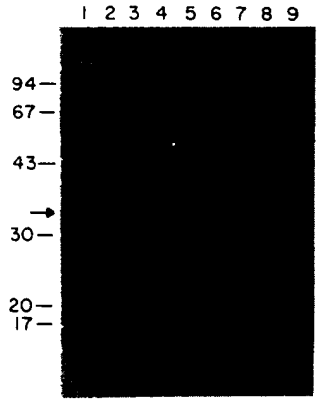

Fig. 8. *Effect of cap analogues on cell-free translation*

HeLa cell-free protein synthesizing extracts were incubated with Sindbis virus mRNA and [^{35}S]methionine for 60 min at 37°C (Sonenberg *et al.*, 1980). Products were analysed by polyacrylamide-gel electrophoresis and autoradiography. Lane 1, no added mRNA; lane 2 approx. 0.3 μg of Sindbis virus mRNA; lanes 3–9 mRNA plus 1.4 μg of affinity purified 24K-CBP. Samples in lanes 4, 5 and 6 also contained m^{7}GMP and in lanes 7, 8 and 9 m^{7}GMP methyl ester at 0.25, 0.1, and 0.05 mM, respectively. Normalized levels of viral capsid protein (arrow, M_r approx. 33000) determined from densitometry tracings of lanes 2–9 were 1.0, 5.5, 0, 0.6, 4.1, 5.3, 4.7 and 6.7, respectively.

prepared by fusing non-producer, P3X63Ag8.653 myeloma cells with spleen cells from mice that had been immunized with 24K-CBP. The antigen was purified by m^{7}GDP-Sepharose affinity chromatography from rabbit erythrocytes rather than from reticulocyte ribosomal high salt wash. This was done in an effort to avoid the

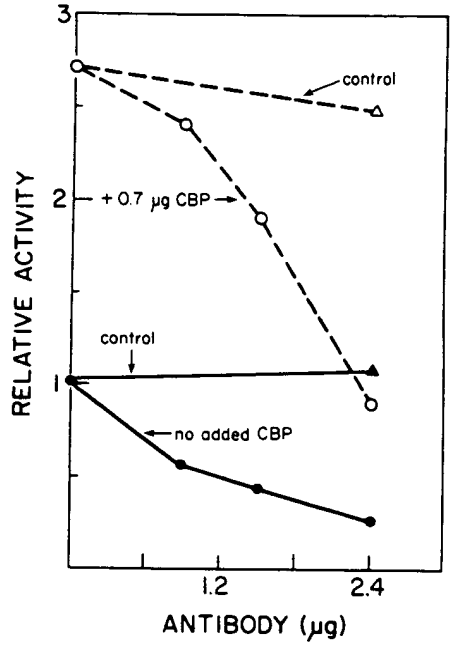

Fig. 9. *Inhibition of capped mRNA translation by anti-(24K-CBP) monoclonal antibody*

Sindbis virus mRNA (approx. 0.3 μg) was translated in 25 μl incubation mixtures (Rose *et al.*, 1978) that included 9 μl of micrococcal nuclease-treated HeLa cell extract, 140 mM-K^+, 0.5 mM-magnesium acetate, 10 μCi of [^{35}S]methionine (Amersham Searle, approx. 1200 Ci/mmol), and, where indicated, 0.7 μg of 24K-CBP purified from rabbit erythrocytes. Hybridoma cells from a clone selected for production of antibody to 24K-CBP were injected intraperitoneally into mice, and 1½ to 2 weeks later the ascites fluid was collected, clarified and made to 40% saturation with solid $(NH_4)_2SO_4$. The precipitated protein was applied to a tandem column (0.7 cm × 8 cm) consisting of equal layers of CM-52 above DE-52 resin equilibrated with phosphate-buffered saline containing 1 mM-EGTA (Palacios *et al.*, 1972). Peak A_{280} fractions were pooled and dialysed exhaustively against 0.1 M-potassium phosphate buffer (pH 8) containing 0.02% NaN_3. The dialysed protein solution ($160\,A_{280}$ units) was applied to a column of protein A-Sepharose (1.5 cm × 4 cm) equilibrated with 0.1 M-potassium phosphate (pH 8) containing 0.02% NaN_3. After washing with 30 ml of the equilibration buffer, samples were eluted sequentially with 40 ml of 0.1 M-sodium citrate (pH 6) followed by the same volume of 0.1 M-sodium citrate (pH 4.5), both containing 0.2% NaN_3. Peak fractions from the pH 6 eluate ($1.3\,A_{280}$ units) and pH 4.5 eluate ($0.8\,A_{280}$ units) were pooled separately and dialysed against 20 mM-Tris/HCl (pH 7.5)/150 mM-KCl. The pH 6 eluate, which was active by enzyme-linked immunoadsorbent (ELISA) assay, was used after concentration to 0.32 mg of protein/ml. The control sample was P3X63 IgG_1 of unknown specificity from ascites fluid of Balb/c mice injected with P3X63 myeloma cells. Protein was precipitated with $(NH_4)_2SO_4$ as above, and after dialysis $12\,A_{280}$ units were applied to protein A-Sepharose. The pH 6 eluate ($1.7\,A_{280}$ units) was dialysed and concentrated to 0.6 mg/ml. Both antibody preparations just before use were treated for 30 min at 37°C with 0.04% diethyl pyrocarbonate (by dilution from a 1% solution in ethanol) in order to eliminate residual ribonuclease. Recently, however, we found that this treatment inactivated the antibody as measured by radioimmunoassay and ELISA assays but apparently not as determined by inhibition of capped mRNA translation. Affinity purified 24K-CBP was pre-incubated for 10 min at room temperature with the purified anti-(24K-CBP) or control immunoglobulin and then added to translation assay mixtures. After incubation at 37°C for 60 min, samples were precipitated (approx. 3 h) with 1 ml of acetone, pelleted by centrifugation, air-dried, dissolved in 20 μl of Laemmli sample buffer (Laemmli, 1970), heated for 5 min at 100°C and analysed by electrophoresis in a 12.5% modified Laemmli slab gel containing a 3% stacking gel. The autoradiogram (Kodak X-Omat film) of the dried gel was traced, and the relative translational activities were determined from densitometry readings.

high molecular weight polypeptides that copurify with 24K-CBP in some conditions (Tahara *et al.*, 1981). The hybridoma clone was grown in the ascites form, and immunoglobulin was purified from the fluid by $(NH_4)_2SO_4$ precipitation and protein A–Sepharose chromatography. High-titre antibodies were not obtained, possibly because of tolerance to a rabbit protein that presumably is related to a putative cap binding protein present in the mouse (and perhaps conserved among other mammals). However, the anti-(24K-CBP) inhibited translation of Sindbis virus capped mRNA in extracts of HeLa cells (Fig. 9). The stimulation of capped mRNA translation obtained by addition of purified 24K-CBP was also antibody sensitive; 2.4 μg of purified antibody/25 μl assay diminished translation to below the unstimulated level of protein synthesis. Control antibodies, e.g. a monoclonal antibody to reovirus cores or an immunoglobulin fraction from mouse ascites cells, had little or no effect. It should be noted that tissue culture fluids from hybridoma cells grown *in vitro* were not used because the growth medium from non-producer parental cells yielded protein fractions that were inhibitory for cell-free translation.

The antibody to cap binding protein was similarly active in mouse L cell and HeLa protein synthesizing extracts. It inhibited [^{35}S]methionine incorporation into Sindbis virus capsid protein in both the unstimulated and 24K-CBP stimulated reactions (Fig. 10). By contrast, a naturally uncapped RNA, encephalomyocarditis (EMC) virus RNA, was translated equally well in HeLa cell extracts in the absence or presence of the antibody. This finding is consistent with the 24K-CBP specificity of the monoclonal antibody and cap-independent expression of EMC virus RNA.

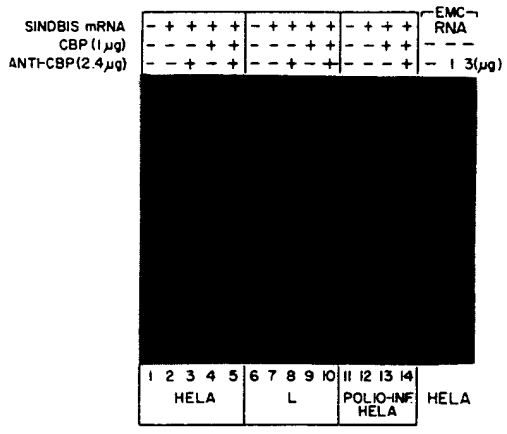

Fig. 10. *Inhibition of mouse and HeLa cell-free translation by anti-CBP antibody*

Gel analysis by autoradiography of [^{35}S]methionine-labelled products directed by Sindbis virus capped mRNA and EMC virus uncapped RNA in uninfected HeLa and L cell extracts and in extracts of poliovirus-infected HeLa cells. Incubation mixtures included, as indicated, approx. 0.1–0.3 μg of Sindbis mRNA or 0.25 μg of EMC virus RNA, 24K-CBP or in the case of extracts of polio-infected cells, CBP II (restoring activity, see Tahara *et al.*, 1981) and/or anti-CBP immunoglobulin. Conditions were as described in the legend to Fig. 9, except that for translation by L cell extracts, the K^+ and magnesium acetate levels were 100 mM and 2 mM, respectively.

Poliovirus, a human picornavirus relative of mouse EMC virus, also contains an uncapped RNA that functions as viral messenger. Infection of HeLa cells with poliovirus results in the shut-off of host (capped) mRNA translation in favour of viral protein formation. The switch to translation of uncapped RNAs is maintained in extracts prepared from poliovirus-infected cells. In such extracts capped mRNA translation can be restored by addition of protein preparations that contain 24K-CBP (Trachsel *et al.*, 1980; Tahara *et al.*, 1981). Restoring activity was decreased by exposure to anti-(24K-CBP), indicative of a functionally important role of cap binding proteins in the initiation of capped mRNA translation (Fig. 10).

Conclusion

Most viral and cellular mRNAs of eukaryotes are 5′-terminally modified by the addition of a cap structure, $m^7G^{5'}ppp^{5'}N$. The presence of a cap has important consequences, most strikingly with respect to the ability of mRNA to initiate protein synthesis effectively. The facilitating effect of cap on translation initiation appears to be mediated by cap binding protein(s) such as a 24000 dalton cap binding protein (24K-CBP) purified from rabbit blood cells. It stimulated capped mRNA translation *in vitro* and could be chemically cross-linked to the 5′ end of oxidized, capped mRNA. Both cap recognition processes were inhibited by an excess of the cap analogue, m^7GMP. By contrast, the methyl ester of m^7GMP was inactive as a cap analogue, suggesting that the interaction of cap binding protein with cap is a highly specific and discriminatory one. Monoclonal antibody prepared against affinity purified 24K-CBP inhibited translation of Sindbis virus capped mRNA but not naturally uncapped EMC virus RNA in extracts of HeLa and mouse L cells. Stimulation of capped mRNA function by 24K-CBP was also prevented by the antibody, as was the restoration of capped mRNA translation by addition of a cap binding protein complex to extract of poliovirus-infected HeLa cells.

The possible regulatory roles of caps and cap binding proteins in gene expression is being explored. Of some interest is the mechanism of enhancement of capped mRNA translation by 24K-CBP. Presumably the effect is related to the finding that 40S ribosomal subunits attach to the 5′ end of mRNA before positioning on the initiator (usually 5′-proximal) AUG triplet during initiation complex formation (Kozak, 1981). Ribosome binding to capped mRNA (unlike naturally uncapped messengers: Kozak, 1980*b*; R. Jackson, personal communication) is an ATP-dependent process (Marcus, 1970; Trachsel *et al.*, 1977; Kozak, 1980*a*, *b*). However, binding of capped mRNA is ATP-independent if the mRNA is unfolded, e.g. substituted with IMP in place of GMP during synthesis (Kozak, 1980*a*; Morgan & Shatkin, 1980) or treated with bisulphite to convert CMP residues to UMP (Kozak, 1980*a*) and thus prevent G·C base-pairing. Furthermore, unlike native mRNAs, binding of the modified, denatured mRNAs is relatively insensitive to inhibition by cap analogues. The combined findings that capped mRNAs depleted of secondary structure have a lower ATP requirement and a decreased dependence on the cap suggests that cap binding protein facilitates ribosome attachment to (and correct positioning on) capped mRNA by an ATP-dependent process. Recently we observed that eukaryotic initiation factors eIF-4A and eIF-4B, two factors that are involved in mRNA binding to ribosomal subunits, can be cross-linked to the 5′ end of

oxidized, capped mRNA in the presence of ATP, Mg^{2+} and 24K-CBP (Grifo *et al.*, 1982). Thus, interaction of mRNA with the cap binding protein may direct binding of other protein factors that are involved in the early stages of protein synthesis initiation.

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